

REGULATION OF IMMUNE RESPONSES BY ATTRACTINBackground of the Invention

5 The present invention relates to agents that regulate immune responses.

Analysis of *in vitro* immune responses allows basic interactions between cells and soluble modulators to be studied, but interpretations may be difficult to extend
10 to actual responses *in vivo*, where reactions occur in complex cellular environments with constant dynamic modification of the extracellular environment. An important role is played by the extracellular matrix which interacts with adhesion structures on the surface
15 of immune cells, directing cell migration, localization and clustering and subsequently influences the activity of local cytokines and lymphokines [Shimizu et al. (1991) *FASEB J.* 5, 2292-2299; Gilat et al. (1996) *Immunol. Today* 17, 16-20]. The passage of activated leukocytes between
20 endothelial cells and their migration through the extracellular matrix to sites of inflammation is facilitated by the upregulated surface expression of several adhesion molecules and proteases [Hauzenberger et al. (1995) *Crit. Rev. Immunol.* 15, 285-316].

25 On activated T cells, one of the most prominently expressed proteases is CD26, which is a marker of T lymphocytes capable of migrating across endothelial barriers [Masuyama et al. (1992) *J. Immunol.* 148, 1367-1374; Brezinschek et al. (1995) *J. Immunol.* 154, 3062-3077] and has a collagen-binding domain [Loster et
30 al. (1995) *Biochem. Biosphys. Res. Commun.* 217, 341-348]. CD26 is now known to be identical to both dipeptidyl peptidase IV (DPPIV) and adenosine deaminase binding protein [Kameoka et al. (1993) *Science* 261, 466-469].
35 The understanding of the multifunctionality of CD26, which is the prototype for a family of related molecules

09/787097

- 2 -

which includes Fibroblast Activation Protein [Scanlan et al. (1994) *Proc. Natl. Acad. Sci. USA* 91, 5657-5661], DPPIV [Wada et al. (1992) *Proc. Natl. Acad. Sci. USA* 89, 197-201] and Seprase [Goldstein et al. (1997) *Biochim. Biophys. Acta* 1361, 11-19; Pineiro-Sanchez et al. (1997) *J. Biol. Chem.* 272, 7595-7601], has expanded to include T lymphocyte costimulatory activity, where it enhances immune responses channeled through the CD3/T cell receptor complex [Dang et al. (1990) *J. Immunol.* 144, 4092-4100].

A soluble serum form of DPPIV had previously been identified [Tanaka et al. (1994) *Proc. Natl. Acad. Sci. USA* 91, 3082-3086], and its circulating levels were determined to be related to the ability of peripheral blood mononuclear cells (PBMC) to react *in vitro* to recall antigens such as tetanus toxoid. Based on this activity, it was conjectured that the identified soluble serum protein was a soluble form of CD26. However, upon purification of the protein, its glycosylated form was found to have a molecular weight of 175 kDa, and therefore, it was distinct from the 105 kDa glycosylated form of DPPIV/CD26 [Duke-Cohan et al. (1995) *J. Biol. Chem.* 270, 14107-14114]. The soluble serum protein having DPPIV activity was designated DPPT-L. DPPT-L appeared to be related to CD26 in that it displayed some CD26 antigenic epitopes, it was rapidly expressed as a T lymphocyte activation antigen, after 48-72 hr it was released from the lymphocyte membrane, and it could upregulate recall antigen-specific T cell responses in a manner similar to that of CD26 [Duke-Cohan et al. (1996) *J. Immunol.* 156, 1714-1721].

Summary of the Invention

The invention features four isolated forms of the human attractin polypeptide. These are soluble

- 3 -

attractin-1 (previously named DPPT-L in the mistaken belief that it was related to, and even a soluble form of, DPPIV/CD26), membrane attractin-1, soluble attractin-2, and membrane attractin-2. Text that refers to

5 attractin without specifying soluble versus membrane or attractin-1 versus attractin-2 is pertinent to all forms of attractin. Membrane attractin differs from soluble attractin in that it has a transmembrane domain and a cytoplasmic domain. Attractin-2 differs from attractin-1

10 in that it contains a 74-amino acid insert in the N-terminal part of the polypeptide. The attractin molecules serve to enhance immune response by promoting macrophage and monocyte spreading in the presence of T cells. The invention also includes nucleic acid

15 molecules encoding attractin polypeptides, vectors containing the nucleic acid molecules, and cells transformed with the vectors. In addition, the invention includes methods of enhancing or inhibiting immune responses and methods of identifying compounds that

20 enhance or inhibit immune responses.

Specifically, the invention features an isolated DNA including: (a) a nucleic acid sequence that encodes a polypeptide that enhances spreading of a macrophage or a monocyte and that hybridizes under stringent conditions

25 to the complement of a sequence that encodes a polypeptide with an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:18; or (b) a complement of this nucleic acid sequence. The nucleic acid sequence

30 included in the isolated DNA will be at least 10 bp, 15 bp, 25 bp, 50 bp, 75 bp, 100 bp, 125 bp, 150 bp, 175 bp, 200 bp, 250 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1000 bp, 1,500 bp, 2,000 bp, 3,000 bp, or 4,000 bp long. The nucleic acid sequence

35 can encode a polypeptide that includes the amino sequence

PCT/US99/20948

- 4 -

of SEQ ID NO:2, SEQ ID NO:10, SEQ ID NO:12, or SEQ ID NO:18. Examples include nucleotide sequences SEQ ID NO:1, SEQ ID NO:11, SEQ ID NO:13, and SEQ ID NO:19.

An isolated polypeptide within the invention can
5 include the amino acid sequence of SEQ ID NO:2, SEQ ID NO:10, SEQ ID NO:12, or SEQ ID NO:18, or can differ from one of these sequences solely by one or more conservative amino acid substitutions. The polypeptides of the invention also embrace fusion proteins containing both
10 (a) an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:18, but lacking methionine at position 1 of said amino acid sequence; and (b) a heterologous leader peptide. Also included are isolated nucleic acid
15 molecules encoding the fusion proteins.

The invention features methods of enhancing spreading of a macrophage or a monocyte *in vitro*. These methods include coculturing a monocyte or a macrophage and a T cell with one or more of the following agents:
20 (a) an isolated attractin polypeptide with the amino acid sequence of SEQ ID NO:2, SEQ ID NO:10, SEQ ID NO:12, or SEQ ID NO:18; (b) a functional fragment of one or more of these attractin polypeptides; or (c) the polypeptide or the functional fragment, but with at least one
25 conservative amino acid substitution.

The above polypeptides and nucleic acids can be used in a method of treating a mammal (e.g., a human) in need of an enhanced immune response. The method includes the step of delivering, to a tissue of a mammal where the
30 tissue contains T cells and macrophages or monocytes, one of the above agents. The method can involve administration of the agent or a nucleic acid encoding the agent to the mammal. The human can be one suspected of being immunodeficient (e.g., one having common
35 variable immunodeficiency) and/or of having cancer; and

- 5 -

can be performed before, during, or after chemotherapy or radiation therapy.

The invention also embodies a method of inhibiting spreading of a macrophage or a monocyte in a mammal. The method includes administering to the mammal an isolated compound that binds to an attractin polypeptide, and interferes with its function. The product can be an antibody and the mammal can be a human, e.g., a human suspected of having an autoimmune disease or a transplant recipient.

The invention also features vectors including any of the isolated DNAs of the invention, e.g. a vector in which the nucleic acid sequence encoding the relevant polypeptide is operably linked to a regulatory element which allows expression of the coding sequence in a cell. Cultured cells including the above vectors can be used in methods of producing any of the polypeptides of the invention. These methods include culturing the appropriate cell and purifying the polypeptide from it.

The invention also features a method of identifying a compound that inhibits an immune response. The method includes: a) providing an isolated polypeptide containing an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:18, or the same amino acid sequence but with one or more conservative amino acid substitutions; b) co-culturing a T cell and a macrophage or a monocyte with the isolated polypeptide and the test compound; c) determining whether the test compound inhibits spreading of the macrophage or the monocyte, as an indication that the test compound inhibits an immune response.

Alternatively, the method can include: a) providing a test compound; b) combining the test compound, a T cell, a macrophage or a monocyte, and the isolated polypeptide; and c) determining whether the test compound enhances

- 6 -

spreading of the macrophage or the monocyte, as an indication that the test compound inhibits an immune response.

Also within the invention is a method of

5 identifying a compound that enhances an immune response. The method includes: a) providing a test compound; b) combining the test compound, a T cell, a macrophage or a monocyte, and an isolated polypeptide of the invention; and c) determining whether the test compound enhances

10 spreading of the macrophage or the monocyte, as an indication that the test compound inhibits an immune response. Alternatively, the method can include: a) providing the isolated polypeptide; b) co-culturing a T cell and a macrophage or a monocyte with the isolated

15 polypeptide and the test compound; c) determining whether the test compound inhibits spreading of the macrophage or the monocyte, as an indication that the test compound inhibits an immune response.

Also within the invention is an antibody (e.g., a

20 scFv) that binds to a polypeptide with the amino acid sequence of SEQ ID NO:10, SEQ ID NO:12, or SEQ ID NO:18, but does not bind to CD26 or to a polypeptide with the sequence of SEQ ID NO:2.

The invention also features an ex vivo method of

25 treating a mammal (e.g., a human patient) in need of an enhanced immune response. The method includes: a) providing a recombinant cell which is the progeny of a cell obtained from the mammal and has been transfected or transformed ex vivo with a nucleic acid encoding an

30 "agent" or a functional fragment of the agent so that the cell expresses the agent or functional fragment; and b) administering the cell to the mammal. The "agent" is:

(i) an attractin polypeptide that includes the amino acid sequence of SEQ ID NO:2, SEQ ID NO:10, SEQ ID NO:12, or

35 SEQ ID NO:18; (ii) a functional fragment of the attractin

PCT/US99/20948

Another aspect of the invention is an isolated functional attractin fragment including at least amino acid residues 31-104 of SEQ ID NO:12 or SEQ ID NO:18, amino acid residues 1279-1301 of SEQ ID NO:12, amino acid residues 1219-1429 of SEQ ID NO:12, or amino acid residues 1302-1429 of SEQ ID NO:12.

The term "isolated" polypeptide or peptide fragment as used herein refers to a polypeptide or a peptide fragment which either has no naturally-occurring counterpart (e.g., a peptidomimetic), or has been substantially separated or purified from components which naturally accompany it, e.g., in tissues such as pancreas, liver, spleen, ovary, testis, muscle, joint tissue, neural tissue, gastrointestinal tissue, or body fluids such as blood, serum, or urine. Typically, the polypeptide or peptide fragment is considered "isolated" when it is at least 70%, by dry weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, a preparation of a polypeptide (or peptide fragment thereof) of the invention is at least 80%, more

- 8 -

preferably at least 90%, and most preferably at least 99%, by dry weight, the polypeptide (or the peptide fragment thereof), respectively, of the invention.

Thus, for example, a preparation of polypeptide x is at least 80%, more preferably at least 90%, and most, preferably at least 99%, by dry weight, polypeptide x. Since a polypeptide that is chemically synthesized is, by its nature, separated from the components that naturally accompany it, a synthetic polypeptide is by definition "isolated."

An isolated polypeptide (or peptide fragment) of the invention can be obtained, for example, by extraction from a natural source (e.g., from human tissues or bodily fluids); by expression of a recombinant nucleic acid encoding the peptide; or by chemical synthesis. A peptide that is produced in a cellular system different from the source from which it naturally originates is "isolated," because it will be separated from components which naturally accompany it. The extent of isolation or purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

An "isolated DNA" means a DNA which either (a) has a non-naturally occurring sequence (e.g., a cDNA from a gene that naturally has introns), or (b) has a naturally occurring (i.e., genomic) sequence, but is free of the genes that flank the sequence in the genome of the organism in which the gene of interest naturally occurs. The term "isolated DNA" therefore includes a recombinant DNA incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote at a site other than the site at which it occurs naturally. It also includes a separate molecule such as a cDNA; a genomic fragment; a fragment produced by polymerase chain reaction (PCR); a

- 9 -

restriction fragment; a DNA encoding a non-naturally occurring mutein, fusion protein, or fragment of a given protein; or a nucleic acid which is a degenerate variant of a naturally occurring nucleic acid. In addition, it
5 includes a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein.

"Spreading" of a macrophage or a monocyte, which occurs after exposure of the macrophage or monocyte to an attractin protein and a T cell, involves flattening of
10 the macrophage or monocyte on a surface, expansion of the macrophage's or monocyte's margins, and an increase in the cell's surface area. The spread macrophage or monocytes may produce cellular processes visible
microscopically. Macrophages and monocytes that have
15 spread can be distinguished from, e.g., fibroblasts or T cells, by their expression of surface CD14.

As used herein, a "fragment" of an attractin polypeptide contains part but not all of the full-length polypeptide. Generally, fragments will be five or more
20 amino acids in length. An antigenic fragment has the ability to be recognized and bound by an antibody.

As used herein, a "functional fragment" of an attractin polypeptide is a fragment of the polypeptide that has the ability to induce spreading of a macrophage
25 or a monocyte in the presence of a T cell. Methods of establishing whether a fragment of an attractin molecule is functional are based upon those described herein for full-length polypeptides. For example, fragments of interest can be made by either recombinant, synthetic, or
30 proteolytic digestive methods. Such fragments can then be isolated and tested for their ability to enhance spreading of macrophages or monocytes by procedures described herein.

As used herein, "operably linked" refers to an
35 expression control sequence (e.g., a promoter, enhancer,

09/09/99 15:09:59

- 10 -

or the like) linked to a coding sequence in a manner that permits the expression control sequence to control expression of the coding sequence.

As used herein, the term "antibody" refers not only to whole antibody molecules, but also to antigen-binding fragments, e.g., Fab, F(ab')₂, Fv, and single chain Fv fragments. Also included are chimeric antibodies.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In case of conflict, the present document, including definitions, will control. Preferred methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention. All publications, patent applications, patents and other references mentioned herein are incorporated by reference in their entirety. The materials, methods, and examples disclosed herein are illustrative only and not intended to be limiting.

Other features and advantages of the invention, e.g., enhancing immune responses in mammalian subjects, will be apparent from the following description, from the drawings and from the claims.

Brief Description of the Drawings

Figs. 1A-1F are photomicrographs showing monocyte spreading and T cell clustering after a 48 hr incubation of peripheral blood mononuclear cells (PBMC) with various concentrations of purified, natural soluble attractin-1. PBMC were incubated with no soluble attractin-1 (Fig. 1A) or soluble attractin-1 at a concentration of 0.5 µg/ml (Fig. 1B), 1µg/ml (Fig. 1C), 2 µg/ml (Fig. 1D), 5 µg/ml

Fig. 2 is a depiction of the amino acid sequence of soluble attractin-1 (SEQ ID NO:2). The sequences identified by N-terminal sequencing of tryptic and chymotryptic peptides are underlined.

Fig. 4A-4C are diagrams showing the organization of soluble attractin-1 cDNA and peptide domains. Fig. 4A is a diagram of soluble attractin-1 cDNA. The bases shown in upper case at the origin represent bases satisfying the Kozak consensus. Fig. 4B shows a comparison of soluble attractin-1 protein domains and motifs with those of *C. elegans* F33C8.1 protein. The horizontal bars depict the position of cysteines shared by both sequences. Fig. 4C shows a comparison of the putative catalytic serine motif of soluble attractin-1 with the catalytic serine motifs of other serine proteases. The shaded box indicates agreement with the consensus, '#' or exclusion from the shaded boxes indicates conflict, and 'X' indicates satisfaction by any amino acid. The parentheses enclose amino acids any of which would satisfy the consensus.

- 12 -

Figs. 5A-5D are photomicrographs showing the intracellular localization of glycosylated soluble attractin-1 by immunogold electron microscopy using rabbit polyclonal antibody specific for soluble attractin-1; in resting T lymphocyte with no evidence of soluble attractin-1 expression (Fig. 5A); in T cells activated for 48 hr with PHA and in which, soluble attractin-1 is expressed in large vacuoles (Fig. 5B); in vesicles in which soluble attractin-1 localizes in an electron dense core (Fig. 5C); and in vesicles containing soluble attractin-1 breaking open at the cell surface, releasing soluble attractin-1 (Fig. 5D).

Figs. 6A-6C are photographs showing expression of recombinant soluble attractin-1 and immunoprecipitation by antibody specific for natural soluble attractin-1. Fig. 6A shows a photograph of an SDS-PAGE gel of soluble attractin-1 transcribed and translated in vitro in the absence or presence of glycosyl transferases. Fig. 6B is a photograph of a Western blot (developed with antibody specific for myc) of lysates of 293T cells transiently transfected with pSecTag2B-soluble attractin-1 or pSecTag2B vector control. Fig. 6C shows a photograph of an SDS-PAGE gel of soluble attractin-1 precipitated with pre-immune serum or polyclonal antibody specific for soluble attractin from lysates of CHO cells stably transfected with pSecTag2B-attractin.

Figs. 7A-7D are photomicrographs showing that recombinant soluble attractin-1 mediates monocyte/macrophage spreading and T cell clustering. Resting PBMC were incubated for 48 hr without soluble attractin-1 (Fig. 7A) or with soluble attractin-1 at a concentration of 1 μ g/ml (Fig. 7B), 2 μ g/ml (Fig. 7C), or 5 μ g/ml (Fig. 7D).

Fig. 8 is a depiction of the nucleotide sequence of soluble attractin-1 cDNA (SEQ ID NO:1).

- 13 -

Fig. 9 is a depiction of the amino acid sequence of membrane attractin-1 (SEQ ID NO:10).

Fig. 10 is a depiction of the nucleotide sequence of membrane attractin-1 encoding cDNA (SEQ ID NO:11).

5 Fig. 11 is a depiction of the amino acid sequence of soluble attractin-2 (SEQ ID NO:18).

Fig. 12 is a depiction of the nucleotide sequence of soluble attractin-2 encoding cDNA (SEQ ID NO:19).

10 Fig. 13 is a depiction of the amino acid sequence of membrane attractin-2 (SEQ ID NO:12).

Fig. 14 is a depiction of the nucleotide sequence of membrane attractin-2 encoding cDNA (SEQ ID NO:13).

Fig. 15 is a series of fluorescence flow cytometric histograms showing the expression of MHC class
15 I, MHC class II, B7.1, B7.2, CD11a, CD29, CD54, and CD58 molecules on the surface of total peripheral blood leukocytes ("All"), lymphocytes ("Lymph"), and monocytes ("Mono"), prior to culture ("DO"), and after a 72 hr
20 incubation of peripheral blood leukocytes with either recombinant soluble attractin-1 ("X") or granulocyte macrophage colony-stimulating factor ("GM-CSF").

Description of the Invention

The invention is based, in part, on the cloning of cDNA molecules encoding different overlapping regions of
25 human soluble attractin-1, membrane attractin-1, soluble attractin-2, and membrane attractin-2. Contrary to initial indications when soluble attractin-1 was first studied, It was determined that there is no significant amino acid sequence homology between attractin and CD26,
30 or any other characterized human protein. Both purified serum-derived and recombinant soluble attractin-1 induce the spreading of macrophages and monocytes that become the focus for the clustering of non-proliferating T lymphocytes. T lymphocytes use soluble attractin-1, at
35 least, to marshall together the cells required to form a

09787597 052704

- 14 -

cluster of co-operating immune cells. Since membrane attractin-1 and the putative attractin-2 molecules contain all the functional domains of soluble attractin, it is likely that they have similar activity. Thus, attractin has an important role in the regulation of the immune response.

The various forms of attractin are encoded by alternatively spliced mRNA molecules transcribed from a single gene. The 134-kDa soluble attractin-1 protein includes a putative serine protease catalytic serine at amino acid residue 26, four EGF-like motifs, a CUB domain, a C-type lectin domain and a domain homologous with the ligand-binding region of the common cytokine receptor γ chain (Fig. 4C). Except for the latter two domains, the overall structure shares high homology with the *C. elegans* F33C8.1 protein, suggesting that attractin has evolved new domains and functions in parallel with the development of cell-mediated immunity. Membrane attractin-1 contains all these domains and, in addition, C-terminal transmembrane and cytoplasmic domains. Furthermore, attractin-2 has a 74 amino acid insertion, immediately after amino acid residue 30 of attractin-1. This insertion is likely to be important as a glycosylation targeting motif (e.g., a Golgi-targeting motif).

The experiments described in Examples 2, 5, and 10 below show that soluble attractin-1 mediates an interaction between T lymphocytes and monocytes, leading to adherence and spreading of the monocytes which become a focus for T lymphocyte clustering. No difference was observed in attractin mRNA expression between resting and activated PBMC, indicating that a regulatory step exists between transcription and glycosylation rather than in induction of *de novo* mRNA synthesis. Despite extensive N-glycosylation of the isolated serum (soluble)

- 15 -

attractin, there are no consensus signal sequences encoded in the cDNA sequences encoding attractin. Another protein may chaperone attractin through the Golgi complex and endoplasmic reticulum. However, several proteins are known to be secreted without a signal peptide, including FGF-9 [Miyamoto et al. (1993) *Mol. Cell. Biol.* 13, 4251-4259], IL1- α and β [Rubartelli et al. (1990) *EMBO J.* 9, 1503-1510], FGF-1 [Tarantini et al. (1995) *J. Biol. Chem.* 270, 29039-29042], FGF-2 [Mignatti et al. (1992) *J. Cell. Physiol.* 151, 81-93], and platelet-derived endothelial cell growth factor [Mignatti et al. (1992) *J. Cell. Physiol.* 151, 81-93]. This has led to the proposal of alternative secretory pathways with slow exocytic release from large cytoplasmic pools [Rubartelli et al. (1997) In *Unusual Secretory Pathways: from Bacteria to Man*, eds. Kuchler et al. (R.G. Landes Co., Austin, TX), pp. 87-114]. The electron microscopy results described above confirm that the early activated T lymphocyte secretion of soluble attractin, at least, results from vesicular release at the plasma membrane.

Expression of attractin on the surface of activated T cells could involve any of the forms of the protein described herein. Thus, for example, surface could be the membrane form of attractin (-1 or -2) bound via its transmembrane domain to the T cell membrane. Alternatively, it could be soluble attractin (-1 or -2) that has been secreted into the milieu of the T cell and then binds via a cell-surface receptor, or via a non-specific hydrophobic interaction, to the T cell. In addition, attractin on the T cell could be soluble attractin (-1 or -2) that is in transit from the cytoplasm to the exterior of the T cell. The invention is not limited by any particular mechanism of T cell surface expression of attractin.

- 16 -

Proteins, such as attractin, containing EGF-like motifs are usually involved in extracellular signalling or cell guidance [Davis, C.G. (1990) *New Biol.* 2, 410-419]. Attractin also contains a motif representing the ligand-binding region of the cytokine receptor common γ chain [D'Andrea et al. (1990) *Curr. Opin. Cell Biol.* 2, 648-651]. In overall structure and organization of domains, attractin most closely resembles the CUB-containing protein BMP1 (bone morphogenic protein-1) which influences cell interactions during development [Li et al. (1996) *Proc. Natl. Acad. Sci. USA* 93, 5127-5130]. The acronym "CUB" derives from the names of the three prototypic proteins (complement component-1 r/s (C1r/s), U-EGF (epidermal growth factor), and BMP-1). The C-type lectin domain recognizes carbohydrate and is characteristic of the selectin family of proteins involved in adhesion of leukocytes to vascular endothelia. This domain is also characteristic of proteins involved in endocytosis for antigen processing in macrophages and dendritic cells [Weis et al. (1996) *Ann. Rev. Biochem.* 65, 441-473].

There is a high level of identity between attractin and the 143 kDa *C. elegans* F33C8.1 protein. The potential γ -chain ligand binding motif and C-type lectin domain present in attractin are missing in the *C. elegans* transcript, suggesting an evolutionary development in which the human form incorporated these new domains in parallel with the development of cell-mediated immunity.

Like CD26, soluble attractin-1 alone is unable to induce cell proliferation, but is able to enhance the proliferative response of PBL to recall antigens such as tetanus toxoid [Duke-Cohan et al. (1995) *J. Biol. Chem.* 270, 14107-14114; Duke-Cohan et al. (1996) *J. Immunol.* 156, 1714-1721]. Therefore, it appears that soluble

05787097 062704

- 17 -

attractin-1, and indeed the other attractin molecules described herein, modulate the interaction between T cells and macrophages and monocytes, permitting more rapid and/or more effective antigen presentation. It is likely that the minimal immunoregulatory unit consists of an antigen-presenting cell which acts as a focus for a cluster of T helper cells and effector cells [Stuhler et al. (1997) *Proc. Natl. Acad. Sci. USA* 94, 622-627]. The association of the three cell types is neither simultaneous nor random. Rather, the antigen-presenting cell clusters first with the helper T cells, and this cluster acts as a focus for recognition by effector cells [Ridge et al. (1998) *Nature* 393, 474-478]. In the absence of antigen, no proliferation occurs in soluble attractin-1-induced clusters of monocytes and T cells, but if a recall antigen such as tetanus toxoid is present, the clustering of cells maximizes the potential response to the antigen. Attractin may regulate local cytokine activity, either by influencing binding and presentation or by proteolytic modification. Soluble attractin-1 has recently been shown to cleave an N-terminal dipeptide which converts full-length RANTES 1-68 (consisting of amino acid residues 1-68), a potent monocyte chemoattractant, to RANTES 3-68 (consisting of residues 3-68), an equally potent inhibitor of monocyte chemotaxis [Proost et al. (1998) *J. Biol. Chem.* 273, 7222-7227]. Soluble attractin-1 has also been found to bind to macrophages and monocytes. It is possible that it is via this binding that attractin, in any of its forms, may regulate the activity of macrophages and monocytes. It could, for example, provide one of two or more requisite signals necessary for the induction of spreading and subsequent enhanced T-cell clustering. Alternatively, it could complement binding of another molecule to a receptor on macrophages/monocytes.

- 18 -

Furthermore, it could form a bridge between T cells and macrophages/monocytes. Since membrane attractin (-1 and -2) has a cytoplasmic domain, it is likely that binding of a putative ligand to an extracellular region of membrane attractin results in signalling to the T cell. It should be understood, however, that the instant invention is not limited by a particular mechanism of action. The concatenation in attractin of domains related to regulation of cell interactions together with domains related to lymphokine/cytokine binding, the rapid upregulation of attractin cell surface expression by activated T cells, and the clear effect upon T cell-monocyte/macrophage association all suggest that attractin, either as a normal circulating serum protein or as a membrane bound protein, plays a significant role in the immune response *in vivo*.

Attractin Nucleic Acid Molecules

The attractin nucleic acid molecules of the invention can be cDNA, genomic DNA, synthetic DNA, or RNA, and can be double-stranded or single-stranded (i.e., either a sense or an antisense strand). Segments of these molecules are also considered within the scope of the invention, and can be produced, for example, by the polymerase chain reaction (PCR) or generated by treatment with one or more restriction endonucleases. A ribonucleic acid (RNA) molecule can be produced by *in vitro* transcription. Preferably, the nucleic acid molecules encode polypeptides that, regardless of length, are soluble under normal physiological conditions the membrane forms would not be soluble.

The nucleic acid molecules of the invention can contain naturally occurring sequences, or sequences that differ from those that occur naturally, but, due to the degeneracy of the genetic code, encode the same

0970303 "062704
F02299" 69769

- 19 -

polypeptide (for example, the polypeptides with SEQ ID NOS:2, 10, 12, and 18). In addition, these nucleic acid molecules are not limited to coding sequences, e.g., they can include some or all of the non-coding sequences that lie upstream or downstream from a coding sequence.

The nucleic acid molecules of the invention can be synthesized (for example, by phosphoramidite-based synthesis) or obtained from a biological cell, such as the cell of a mammal. Thus, the nucleic acids can be those of a human, non-human primate (e.g., monkey) mouse, rat, guinea pig, cow, sheep, horse, pig, rabbit, dog, or cat.

In addition, the isolated nucleic acid molecules of the invention encompass segments that are not found as such in the natural state. Thus, the invention encompasses recombinant nucleic acid molecules, (for example, isolated nucleic acid molecules encoding any of the forms of attractin described herein) incorporated into a vector (for example, a plasmid or viral vector) or into the genome of a heterologous cell (or the genome of a homologous cell, at a position other than the natural chromosomal location). Recombinant nucleic acid molecules and uses therefor are discussed further below.

Certain nucleic acid molecules of the invention are antisense molecules or are transcribed into antisense molecules. These can be used, for example, to down-regulate translation of attractin mRNA within a cell.

Techniques associated with detection or regulation of genes are well known to skilled artisans and such techniques can be used to diagnose and/or treat disorders associated with aberrant attractin expression. Nucleic acid molecules of the invention are discussed further below in the context of their therapeutic utility.

An attractin family gene or protein can be identified based on its similarity to the relevant

09707057 053701
09707057 053701

- 20 -

attractin gene or protein, respectively. For example, the identification can be based on sequence identity. The invention features isolated nucleic acid molecules which are at least 50% (or 55%, 65%, 75%, 85%, 95%, or 98%) identical to: (a) a nucleic acid molecule that encodes the polypeptide of SEQ ID NO:2, 10, 12, or 18; (b) the nucleotide sequence of SEQ ID NO:1, 11, 13, or 19; or (c) a nucleic acid molecule which includes a segment of at least: (i) 30 (e.g., at least 50, 60, 100, 125, 150, 175, 200, 250, 300, 325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 2000, 3000, or 3540) nucleotides of SEQ ID NO:1; (ii) 30 (e.g., at least 50, 60, 100, 125, 150, 175, 200, 250, 300, 325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 2000, 3000, 4000, or 4050) nucleotides of SEQ ID NO:11; (iii) 30 (e.g., at least 50, 60, 100, 125, 150, 175, 200, 250, 300, 325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 2000, 3000, 4000, or 4250) nucleotides of SEQ ID NO:13; or (iv) 30 (e.g., at least 50, 60, 100, 125, 150, 175, 200, 250, 300, 325, 350, 375, 400, 425, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 2000, 3000, or 3800) nucleotides of SEQ ID NO:19. The invention also features nucleic acid molecules which include a nucleotide sequence encoding a polypeptide that is at least 65% (e.g., at least 70%, 75%, 85%, 95%, or 98%) identical to the amino acid sequence of SEQ ID NO:2, 10, 12, or 18.

The determination of percent identity between two sequences is accomplished using the mathematical algorithm of Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 90, 5873-5877, 1993. Such an algorithm is incorporated into the BLASTN and BLASTP programs of Altschul et al. (1990) *J. Mol. Biol.* 215, 403-410. BLAST nucleotide searches are performed with the BLASTN program, score = 100, wordlength = 12 to obtain

- 21 -

nucleotide sequences homologous to attractin-encoding nucleic acids. BLAST protein searches are performed with the BLASTP program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to attractin. To obtain
5 gapped alignments for comparative purposes, Gapped BLAST is utilized as described in Altschul et al. (1997) *Nucleic Acids Res.* 25, 3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) are used
10 (See <http://www.ncbi.nlm.nih.gov>).

Hybridization can also be used as a measure of homology between two nucleic acid sequences. An attractin-encoding nucleic acid sequence, or a portion thereof, can be used as hybridization probe according to
15 standard hybridization techniques. The hybridization of an attractin probe to DNA from a test source (e.g., a mammalian cell) is an indication of the presence of attractin DNA in the test source. Hybridization conditions are known to those skilled in the art and can
20 be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y., 6.3.1-6.3.6, 1991. Moderate hybridization conditions are defined as equivalent to hybridization in 2X sodium chloride/sodium citrate (SSC) at 30°C, followed by one or more washes in 1 X SSC, 0.1%
25 SDS at 50-60°C. Highly stringent conditions are defined as equivalent to hybridization in 6X sodium chloride/sodium citrate (SSC) at 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C.

The invention also encompasses: (a) vectors that
30 contain any of the foregoing attractin-related coding sequences and/or their complements (that is, "antisense" sequence); (b) expression vectors that contain any of the foregoing attractin-related coding sequences operatively associated with any transcriptional/translational
35 regulatory elements (examples of which are given below)

09767097.00204

- 22 -

necessary to direct expression of the coding sequences;
(c) expression vectors containing, in addition to
sequences encoding an attractin polypeptide, nucleic acid
sequences that are unrelated to nucleic acid sequences
5 encoding attractin, such as molecules encoding a
reporter, marker, or a signal peptide, e.g., fused to
attractin; and (d) genetically engineered host cells that
contain any of the foregoing expression vectors and
thereby express the nucleic acid molecules of the
10 invention.

Recombinant nucleic acid molecules can contain a
sequence encoding a soluble attractin membrane attractin,
or attractin having an heterologous signal sequence. The
full length attractin polypeptide, a domain of attractin,
15 or a fragment thereof may be fused to additional
polypeptides, as described below. Similarly, the nucleic
acid molecules of the invention can encode the mature
form of attractin or a form that includes an exogenous
polypeptide which facilitates secretion.

20 The transcriptional/translational regulatory
elements referred to above and which are further
described below, include, but are not limited to,
inducible and non-inducible promoters, enhancers,
operators and other elements, which are known to those
25 skilled in the art, and which drive or otherwise regulate
gene expression. Such regulatory elements include but
are not limited to the cytomegalovirus hCMV immediate
early gene, the early or late promoters of SV40
adenovirus, the lac system, the trp system, the TAC
30 system, the TRC system, the major operator and promoter
regions of phage A, the control regions of fd coat
protein, the promoter for 3-phosphoglycerate kinase, the
promoters of acid phosphatase, and the promoters of the
yeast α -mating factors.

35 Similarly, the nucleic acid can form part of a

T 0 4 2 9 3 " 4 5 9 2 9 2 6 6

- 23 -

hybrid gene encoding additional polypeptide sequences, for example, sequences that function as a marker or reporter. Examples of marker or reporter genes include β -lactamase, chloramphenicol acetyltransferase (CAT),

5 adenosine deaminase (ADA), aminoglycoside phosphotransferase (neo^r, G418^r), dihydrofolate reductase (DHFR), hygromycin-B-phosphotransferase (HPH), thymidine kinase (TK), lacZ (encoding β -galactosidase), and

10 xanthine guanine phosphoribosyltransferase (XGPRT). As with many of the standard procedures associated with the practice of the invention, skilled artisans will be aware of additional useful reagents, for example, additional

15 sequences that can serve the function of a marker or reporter. Generally, the hybrid polypeptide will include a first portion and a second portion; the first portion being an attractin polypeptide and the second portion being, for example, the reporter described above or an immunoglobulin constant region.

The expression systems that may be used for

20 purposes of the invention include, but are not limited to, microorganisms such as bacteria (for example, *E. coli* and *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA, or cosmid DNA expression vectors containing the nucleic acid molecules of the

25 invention; yeast (for example, *Saccharomyces* and *Pichia*) transformed with recombinant yeast expression vectors containing the nucleic acid molecules of the invention (preferably containing the nucleic acid sequence encoding attractin (contained within SEQ ID NOS:2, 10, 12, or 18);

30 insect cell systems infected with recombinant virus expression vectors (for example, baculovirus) containing the nucleic acid molecules of the invention; plant cell systems infected with recombinant virus expression vectors (for example, cauliflower mosaic virus (CaMV) and

35 tobacco mosaic virus (TMV)) or transformed with

- 24 -

recombinant plasmid expression vectors (for example, Ti plasmid) containing attractin nucleotide sequences; or mammalian cell systems (for example, COS, CHO, BHK, 293, VERO, HeLa, MDCK, WI38, and NIH 3T3 cells) harboring

5 recombinant expression constructs containing promoters derived from the genome of mammalian cells (for example, the metallothionein promoter) or from mammalian viruses (for example, the adenovirus late promoter and the vaccinia virus 7.5K promoter). Also useful as host cells

10 are primary or secondary cells obtained directly from a mammal, transfected with a plasmid vector or infected with a viral vector.

Polypeptides and Polypeptide Fragments

The polypeptides of the invention include soluble

15 attractin-1 and -2, membrane attractin-1 and -2, and functional fragments of these polypeptides. The polypeptides embraced by the invention also include fusion proteins which contain either full-length attractin (any of the forms) or a functional fragment of

20 it fused to unrelated amino acid sequence. The unrelated sequences can be additional functional domains or signal peptides. Signal peptides are described in greater detail and exemplified below.

The polypeptides can be purified from natural

25 sources (e.g., blood, serum plasma, tissues or cells such as T cells or any cell that naturally produces attractin). Smaller peptides (less than 50 amino acids long) can also be conveniently synthesized by standard chemical means. In addition, both polypeptides and

30 peptides can be produced by standard in vitro recombinant DNA techniques and in vivo recombination/genetic recombination (e.g., transgenesis), using the nucleotide sequences encoding the appropriate polypeptides or peptides. Methods well known to those skilled in the art

09787097 "062704

- 25 -

can be used to construct expression vectors containing relevant coding sequences and appropriate transcriptional/translational control signals. See, for example, the techniques described in Sambrook et al.,

- 5 Molecular Cloning: A Laboratory Manual (2nd Ed.) [Cold Spring Harbor Laboratory, N.Y., 1989], and Ausubel et al., Current Protocols in Molecular Biology, [Green Publishing Associates and Wiley Interscience, N.Y., 1989].

- 10 Polypeptides and fragments of the invention also include those described above, but modified for in vivo use by the addition, the amino- and/or carboxyl-terminal ends, of a blocking agent to facilitate survival of the relevant polypeptide in vivo. This can be useful in
- 15 those situations in which the peptide termini tend to be degraded by proteases prior to cellular uptake. Such blocking agents can include, without limitation, additional related or unrelated peptide sequences that can be attached to the amino and/or carboxyl terminal
- 20 residues of the peptide to be administered. This can be done either chemically during the synthesis of the peptide or by recombinant DNA technology by methods familiar to artisans of average skill.

- Alternatively, blocking agents such as
- 25 pyroglutamic acid or other molecules known in the art can be attached to the amino and/or carboxyl terminal residues, or the amino group at the amino terminus or carboxyl group at the carboxyl terminus can be replaced with a different moiety. Likewise, the peptides can be
- 30 covalently or noncovalently coupled to pharmaceutically acceptable "carrier" proteins prior to administration.

- Also of interest are peptidomimetic compounds that are designed based upon the amino acid sequences of the functional peptide fragments. Peptidomimetic compounds
- 35 are synthetic compounds having a three-dimensional

09787097 "062701

- 26 -

conformation (i.e., a "peptide motif") that is substantially the same as the three-dimensional conformation of a selected peptide. The peptide motif provides the peptidomimetic compound with the ability to induce macrophage/monocyte spreading in a manner qualitatively identical to that of the attractin functional peptide fragment from which the peptidomimetic was derived. Peptidomimetic compounds can have additional characteristics that enhance their therapeutic utility, such as increased cell permeability and prolonged biological half-life.

The peptidomimetics typically have a backbone that is partially or completely non-peptide, but with side groups that are identical to the side groups of the amino acid residues that occur in the peptide on which the peptidomimetic is based. Several types of chemical bonds, e.g., ester, thioester, thioamide, retroamide, reduced carbonyl, dimethylene and ketomethylene bonds, are known in the art to be generally useful substitutes for peptide bonds in the construction of protease-resistant peptidomimetics.

Methods of Therapy

The methods of the invention involve combining a macrophage/monocyte, an attractin molecule of the invention, and a T cell, in order to induce spreading of macrophages/monocytes. The T cell can be a CD4+ T cell or a CD8+ T cell. The attractin molecule can be added to the solution containing the cells or it can be expressed on the surface of a T cell, e.g., the T cell that is added to the combined attractin and macrophages/monocytes. The methods can be performed *in vitro*, *in vivo*, or *ex vivo*. *In vitro* application of attractin can be useful, for example, in basic scientific studies of immune mechanisms or for production of

05787097 "062701

- 27 -

macrophages with increased ability to activate T cells for use in studies on macrophage/monocyte function. Furthermore, attractin could be added to in vitro assays (e.g., in T cell proliferation assays) designed to test for immunity to an antigen of interest in a subject from which the T cells were obtained. Addition of attractin to such assays would be expected to result in a more potent, and therefore more readily detectable, in vitro response. However, the methods of the invention will preferably be in vivo or ex vivo (see below).

The attractin proteins and variants thereof are generally useful as immune response-stimulating therapeutics, as described in International Application No. WO 96/38550 (published December 5, 1996), which is incorporated by reference herein in its entirety. For example, the polypeptides of the invention can be used for treatment of disease conditions characterized by immunosuppression: e.g., cancer, AIDS or AIDS-related complex, other virally or environmentally-induced conditions, and certain congenital immune deficiencies. The compounds may also be employed to increase immune function that has been impaired by the use of radiotherapy of immunosuppressive drugs such as certain chemotherapeutic agents, and therefore are particularly useful when given in conjunction with such drugs or radiotherapy. These methods of the invention can be applied to a wide range of species, e.g., humans, non-human primates, horses, cattle, pigs, sheep, goats, dogs, cats, rabbits, guinea pigs, hamsters, rats, and mice.

In Vivo Approaches

In one in vivo approach, the attractin polypeptide (or a functional fragment thereof) itself is administered to the subject. Generally, the compounds of the invention will be suspended in a pharmaceutically-

- 28 -

acceptable carrier (e.g., physiological saline) and administered orally or by intravenous infusion, or injected subcutaneously, intramuscularly, intraperitoneally, intrarectally, intravaginally, intranasally, intragastrically, intratracheally, or intrapulmonarily. They are preferably delivered directly to an appropriate lymphoid tissue (e.g. spleen, lymph node, or mucosal-associated lymphoid tissue (MALT)). The dosage required depends on the choice of the route of administration, the nature of the formulation, the nature of the patient's illness, the subject's size, weight, surface area, age, and sex, other drugs being administered, and the judgment of the attending physician. Suitable dosages are in the range of 0.01-100.0 $\mu\text{g/kg}$. Wide variations in the needed dosage are to be expected in view of the variety of polypeptides and fragments available and the differing efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by i.v. injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization as is well understood in the art. Administrations can be single or multiple (e.g., 2- or 3-, 4-, 6-, 8-, 10-, 20-, 50-, 100-, 150-, or more fold). Encapsulation of the polypeptide in a suitable delivery vehicle (e.g., polymer microparticles or implantable devices) may increase the efficiency of delivery, particularly for oral delivery.

Alternatively, a polynucleotide containing a nucleic acid sequence encoding the attractin polypeptide or functional fragment can be delivered to an appropriate cell of the animal. Expression of the coding sequence will preferably be directed to lymphoid tissue of the subject by, for example, delivery of the polynucleotide

- 29 -

to the lymphoid tissue. This can be achieved by, for example, the use of a polymeric, biodegradable microparticle or microcapsule delivery vehicle, sized to optimize phagocytosis by phagocytic cells such as macrophages. For example, PLGA (poly-lacto-co-glycolide) microparticles approximately 1-10 μm in diameter can be used. The polynucleotide is encapsulated in these microparticles, which are taken up by macrophages and gradually biodegraded within the cell, thereby releasing the polynucleotide. Once released, the DNA is expressed within the cell. A second type of microparticle is intended not to be taken up directly by cells, but rather to serve primarily as a slow-release reservoir of nucleic acid that is taken up by cells only upon release from the micro-particle through biodegradation. These polymeric particles should therefore be large enough to preclude phagocytosis (i.e., larger than 5 μm and preferably larger than 20 μm). Microparticles useful for nucleic acid delivery, methods for making them, and methods of use are described in greater detail in U.S. Patent No. 5,783,567, incorporated herein by reference in its entirety.

Another way to achieve uptake of the nucleic acid is using liposomes, prepared by standard methods. The vectors can be incorporated alone into these delivery vehicles or co-incorporated with tissue-specific antibodies. Alternatively, one can prepare a molecular conjugate composed of a plasmid or other vector attached to poly-L-lysine by electrostatic or covalent forces. Poly-L-lysine binds to a ligand that can bind to a receptor on target cells [Cristiano et al. (1995), *J. Mol. Med.* 73, 479]. Alternatively, lymphoid tissue specific targeting can be achieved by the use of lymphoid tissue-specific transcriptional regulatory elements (TRE) such as a B lymphocyte, T lymphocyte, or dendritic cell specific TRE. Lymphoid tissue specific TRE are known

- 30 -

[Thompson et al. (1992), *Mol. Cell. Biol.* 12, 1043-1053; Todd et al. (1993), *J. Exp. Med.* 177, 1663-1674; Penix et al. (1993), *J. Exp. Med.* 178, 1483-1496]. Delivery of "naked DNA" (i.e., without a delivery vehicle) to an intramuscular, intradermal, or subcutaneous site, is another means to achieve *in vivo* expression.

In the relevant polynucleotides (e.g., expression vectors) the nucleic acid sequence encoding the attractin polypeptide or functional fragment of interest with an initiator methionine and optionally a targeting sequence is operatively linked to a promoter or enhancer-promoter combination.

Short amino acid sequences can act as signals to direct proteins to specific intracellular compartments. For example, hydrophobic signal peptides (e.g., MAISGVPVLGFFIIAVLMSAQESWA (SEQ ID NO:14)) are found at the amino terminus of proteins destined for the ER. While the sequence KFERQ (SEQ ID NO:15) (and other closely related sequences) is known to target intracellular polypeptides to lysosomes, other sequences (e.g., MDDQRDLISNNEQLP (SEQ ID NO:16) direct polypeptides to endosomes. In addition, the peptide sequence KDEL (SEQ ID NO:17) has been shown to act as a retention signal for the ER. Each of these signal peptides, or a combination thereof, can be used to traffic the attractin polypeptides or functional fragments of the invention as desired. DNAs encoding the attractin polypeptides or functional fragments containing targeting signals will be generated by PCR or other standard genetic engineering or synthetic techniques. Targeting sequences are described in greater detail in U.S. Patent No. 5,827,516, incorporated herein by reference in its entirety.

A promoter is a TRE composed of a region of a DNA molecule, typically within 100 basepairs upstream of the point at which transcription starts. Enhancers provide

- 31 -

expression specificity in terms of time, location, and level. Unlike a promoter, an enhancer can function when located at variable distances from the transcription site, provided a promoter is present. An enhancer can also be located downstream of the transcription initiation site. To bring a coding sequence under the control of a promoter, it is necessary to position the translation initiation site of the translational reading frame of the peptide or polypeptide between one and about fifty nucleotides downstream (3') of the promoter. The coding sequence of the expression vector is operatively linked to a transcription terminating region.

Suitable expression vectors include plasmids and viral vectors such as herpes viruses, retroviruses, vaccinia viruses, attenuated vaccinia viruses, canary pox viruses, adenoviruses and adeno-associated viruses, among others.

Polynucleotides can be administered in a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are biologically compatible vehicles which are suitable for administration to a human, e.g., physiological saline. A therapeutically effective amount is an amount of the polynucleotide which is capable of producing a medically desirable result in a treated animal. As is well known in the medical arts, the dosage for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosages will vary, but a preferred dosage for administration of polynucleotide is from approximately 10^6 to 10^{12} copies of the polynucleotide molecule. This dose can be repeatedly administered, as needed. Routes of administration can be any of those listed above.

- 32 -

Ex Vivo Approaches

Peripheral blood leukocytes can be withdrawn from the patient or a suitable donor and treated ex vivo with the attractin protein or polypeptide fragment (whether in
5 soluble form or attached to a solid support by standard methodologies). The leukocytes containing newly-activated monocytes are then introduced into the same or a different patient.

An alternative ex vivo strategy can involve
10 transfecting or transducing cells obtained from the subject with a polynucleotide encoding an attractin polypeptide or functional fragment-encoding nucleic acid sequences described above. The transfected or transduced cells are then returned to the subject. While such cells
15 would preferably be lymphoid cells, they could also be any of a wide range of types including, without limitation, fibroblasts, bone marrow cells, macrophages, monocytes, dendritic cells, epithelial cells, endothelial cells, keratinocytes, or muscle cells in which they act
20 as a source of the attractin polypeptide or functional fragment for as long as they survive in the subject. The use of lymphoid cells would be particular advantageous in that such cells would be expected to home to lymphoid tissue (e.g., lymph nodes or spleen) and thus the
25 attractin polypeptide or functional fragment would be produced in high concentration at the site where they exert their effect, i.e., enhancement of an immune response. In addition, if T cells are used, the T cell expressing the exogenous attractin molecule can be the
30 T cell that is required, together with attractin, to induce spreading and activation of macrophages or monocytes. The attractin can be secreted by the T cell or expressed on the surface of the T cell. The same genetic constructs and trafficking sequences described

- 33 -

for the in vivo approach can be used for this ex vivo strategy.

The ex vivo methods include the steps of harvesting cells from a subject, culturing the cells, transducing them with an expression vector, and maintaining the cells under conditions suitable for expression of the attractin polypeptide or functional fragment. These methods are known in the art of molecular biology. The transduction step is accomplished by any standard means used for ex vivo gene therapy, including calcium phosphate, lipofection, electroporation, viral infection, and biolistic gene transfer. Alternatively, liposomes or polymeric microparticles can be used. Cells that have been successfully transduced are then selected, for example, for expression of the coding sequence or of a drug resistance gene. The cells may then be lethally irradiated (if desired) and injected or implanted into the patient.

20 Methods of Screening for Compounds that Inhibit or Enhance Immune Responses.

The invention provides methods for testing compounds (small molecules or macromolecules) that inhibit or enhance an immune response. Such a method could involve, e.g., culturing macrophages or monocytes with: (a) any of the attractin molecules of the invention, (b) T cells; and (c) a candidate compound. The attractin molecule can be in solution or membrane bound (e.g., expressed on the surface of the T cells) and it can be natural or recombinant. Furthermore, it can be a functional fragment of an attractin molecule. Compounds that inhibit macrophage or monocyte spreading will likely be compounds that inhibit an immune response

05707097, 0570704

- 34 -

while those that enhance macrophage and monocyte spreading will likely be compounds that enhance an immune response. Instead of testing for an effect of a compound on macrophage or monocyte spreading, the ability of the compound to inhibit or enhance induction of B7.1 or MHC class II molecule expression on the macrophage could also be measured.

The invention also relates to using attractin or functional fragments thereof to screen for immunomodulatory compounds that can interact with attractin. One of skill in the art would know how to use standard molecular modeling or other techniques to identify small molecules that would bind to the unique sites of attractin described herein. On such example is provided in Broughton (1997) Curr. Opin. Chem. Biol. 1, 392-398.

A candidate compound whose presence requires at least 1.5-fold (e.g., 2-fold, 4-fold, 6-fold, 10-fold, 150-fold, 1000-fold, 10,000-fold, or 100,000-fold) more attractin in order to achieve macrophage or monocyte spreading than in the absence of the compound can be useful for inhibiting an immune response. On the other hand, a candidate compound whose presence requires at least 1.5 fold (e.g., 2-fold, 4-fold, 6-fold, 10-fold, 100-fold, 1000-fold, 10,000 fold, or 100,000-fold) less attractin to achieve macrophage or monocyte spreading than in the absence of the compound can be useful for enhancing an immune response. Compounds capable of interfering with or modulating attractin function are good candidates for immunosuppressive immunoregulatory agents, e.g., to modulate an autoimmune response or suppress allogeneic or xenogeneic graft rejection.

- 35 -

Attractin Antibodies

The invention features antibodies that bind to any or all of the described attractin polypeptides or fragments of such polypeptides. Such antibodies can be polyclonal antibodies present in the serum or plasma of animals (e.g., mice, rabbits, rats, guinea pigs, sheep, horses, goats, cows, or pigs) which have been immunized with the relevant attractin polypeptide or peptide fragment using methods, and optionally adjuvants, known in the art. Such polyclonal antibodies can be isolated from serum or plasma by methods known in the art. Monoclonal antibodies that bind to the above polypeptides or fragments are also embodied by the invention. Methods of making and screening monoclonal antibodies are well known in the art.

Once the desired antibody-producing hybridoma has been selected and cloned, the resultant antibody can be produced in a number of methods known in the art. For example, the hybridoma can be cultured in vitro in a suitable medium for a suitable length of time, followed by the recovery of the desired antibody from the supernatant. The length of time and medium are known or can be readily determined.

Additionally, recombinant antibodies specific for attractin, such as chimeric and humanized monoclonal antibodies comprising both human and non-human portions, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example, using methods described in Robinson et al., International Patent Publication PCT/US86/02269; Akira et al., European Patent Application 184,187; Taniguchi, European Patent Application 171,496; Morrison et al., European Patent Application 173,494; Neuberger et al., PCT Application WO 86/01533; Cabilly et al., U.S. Patent No. 4,816,567;

- 36 -

Cabilly et al., European Patent Application 125,023;
Better et al. (1988) *Science* 240, 1041-43; Liu et al.
(1987) *J. Immunol.* 139, 3521-26; Sun et al. (1987) *PNAS*
84, 214-18; Nishimura et al. (1987) *Canc. Res.* 47,
5 999-1005; Wood et al. (1985) *Nature* 314, 446-49; Shaw et
al. (1988) *J. Natl. Cancer Inst.* 80, 1553-59; Morrison,
(1985) *Science* 229, 1202-07; Oi et al. (1986)
BioTechniques 4, 214; Winter, U.S. Patent No. 5,225,539;
Jones et al. (1986) *Nature* 321, 552-25; Veroeyan et al.
10 (1988) *Science* 239, 1534; and Beidler et al. (1988) *J.*
Immunol. 141, 4053-60.

Also included within the scope of the present
invention are antibody fragments and derivatives which
contain at least the functional portion of the antigen
15 binding domain of an antibody that binds specifically to
attractin. Antibody fragments that contain the binding
domain of the molecule can be generated by known
techniques. For example, such fragments include, but are
not limited to: F(ab'), fragments which can be produced by
20 pepsin digestion of antibody molecules; Fab fragments
which can be generated by reducing the disulfide bridges
of F(ab'), fragments; and Fab fragments which can be
generated by treating antibody molecules with papain and
a reducing agent. See, e.g., National Institutes of
25 Health, 1 Current Protocols In Immunology, Coligan et
al., ed. §§ 2.8, 2.10 (Wiley Interscience, 1991).
Antibody fragments also include Fv (e.g., single chain Fv
(scFv)) fragments, i.e., antibody products in which there
are no constant region amino acid residues. Such
30 fragments can be produced, for example, as described in
U.S. Patent No. 4,642,334.

The following examples are meant to illustrate,
not limit, the invention.

09787097 062704
T02290 25029250

- 37 -

Example 1. Materials and Methods

Cell techniques: PBMC, erythrocyte rosetting (E⁺) T cells and erythrocyte non-rosetting (E⁻) monocytes/B cells were purified as described previously [Morimoto et al. (1985) *J. Immunol.* 134, 3762-3769]. CHO (dhfr⁻) cells and the Jurkat T cell line were obtained from the American Type Culture Collection (ATCC, Manassas, VA). 293T cells were obtained from Dr. B. Mayer (Children's Hospital, Boston, MA). All cell lines were maintained in RPMI 1640 containing 10% fetal bovine serum. For assessing the biological effects of soluble attractin-1, leukocytes were cultured in serum-free AIM V medium (Life Technologies Inc., Gaithersburg, MD) in 48-well plates (Costar, Cambridge, M). For cell activation, E⁺ lymphocytes (10⁶/ml) supplemented with 0.1% E⁻ cells were incubated in AIM V medium together with phytohemagglutinin (PHA, 1 µg/ml; Murex, Dartford, U.K.) for 48 hr. Cell proliferation was assessed using [³H]-thymidine incorporation as described previously [Duke-Cohan et al. (1995) *J. Biol. Chem.* 270, 14107-14114].

RNA/DNA preparation and analysis: mRNA was isolated using the Poly(A)Pure kit (Ambion, Austin, TX). Northern blots were prepared using standard denaturing formaldehyde agarose electrophoresis techniques and transferred to GeneScreen Plus (NEN-Dupont, Boston, MA). The EST clone R84298 was obtained from the I.M.A.G.E. consortium (Lennon et al. (1996) *Genomics* 33, 151-152) through the ATCC. Both fetal liver cDNA libraries (λgt11 and Marathon cDNA) were obtained from Clontech (Palo Alto, CA). The J5DC T cell library was prepared from 48 hr PHA-activated T lymphocyte mRNA using the Superscript Choice system (Life Technologies Inc.) and ligated into pCDNAI/Amp (Invitrogen, Carlsbad, CA). The GF activated T cell library was prepared and ligated into pCDM8 as described previously [Hall et al. (1996) *Proc.*

05707057 0570704

- 38 -

Natl. Acad. Sci. USA 93, 11780-11785]. The expression vectors, pRc/CMV and pSecTag2B were obtained from Invitrogen. All labeling of DNA probes with ³²P-dCTP was by random priming (Life Technologies Inc.).

5 **Isolation of cDNA encoding soluble attractin-1:**

Tryptic/chymotryptic peptides were prepared and analyzed as described previously [Duke-Cohan et al. (1995) J. Biol. Chem. 270, 14107-14114]. A multiple human hematopoietic tissue Northern blot (Clontech) was
10 screened using the 1.2 kb MfeI/EcoNI R84298 EST. The λgt11-fetal liver library was screened using the 1.3 kb ClaI/HindIII R84298 EST which yielded the pks-43 (4kb) fragment. Hind III digestion of pks-43 released a 5' 982
15 pb fragment which was used to rescreen the fetal liver library and a further 5' sequence, including the putative start codon, was identified (pks-43-1). In order to produce full length recombinant soluble attractin-1, PCR-generated fragments encoding soluble attractin-1 were
20 cloned into the expression vector, pRc/CMV. Using the pks-43-1 as template, a PCR (PCR1) was carried using the following primers: CCCAAGCTTGGGATGGGTGTCGGGCTCAGCCCGC - forward (SEQ ID NO:3) and, ATAAGAATGCGGCGCTAAACTCATTGTTTCAGTTTCGACCTG - reverse (SEQ ID NO:4). A second PCR (PCR2) using the pks-43 fragment
25 as template was carried out using the following primers: CCCAAGCTTGGGATGGTGGCCGCAGCGGCGGC - forward, (SEQ ID NO:5) and CCAGGTCCATCTGTACAAACCCAG - reverse (SEQ ID NO:6). The fragment obtained from PCR1 was digested with HindIII and NaeI and that from PCR2 was digested with NaeI and
30 NotI. The two digested fragments were then cloned together into HindIII/Not-I-digested pRc/CMV. For production of recombinant soluble attractin-1 with disabled start and stop codons into pSecTag2B, a 3.5 kb fragment was amplified from pRc/CMV-attractin using the
35 Advantage GC cDNA PCR system (Clontech) and the primers

097097 "062701

- 39 -

GTGCGTGAAGCTTGTACCGGCAACTGAGGCAAGGCTGA - forward (SEQ ID NO:7) and GTAGTTTTTAAGTCCACGTTTGACTTCGCCGGCGTGCGTG - reverse (SEQ ID NO:8), digested with Hind II/Not I, and ligated into pSecTag2B.

5 **Expression of functional soluble attractin-1:** The Quick TnT system together with canine microsomal membranes (Promega, Madison, WI) was used for *in vitro* transcription and translation. 293T cells were transfected transiently using pSecTag2B-soluble attractin
10 complexed with Lipofectamine Plus (Life Technologies Inc.) and assayed for soluble attractin-1 expression at 48 hr. For Western blotting experiments, cells were lysed in boiling SDS-PAGE sample buffer (x2) and samples run on SDS-PAGE gels, transferred to nitrocellulose by
15 electroblotting, and the membranes were blocked with Tris-buffered saline containing 0.1% Tween 20 and 1% BSA. Blots were incubated with murine antibody specific for myc (1:5000; Amersham, Arlington Heights, IL) or with a horseradish peroxidase (HRP) monoclonal antibody specific
20 for myc (1:2000; Invitrogen) and detected using the Phototope chemiluminescent system (New England Biolabs). For immunoprecipitation experiments, the cells were solubilized in lysis buffer (1% Triton X-1000, 0.1% NP-40, 150 mM NaCl). Lysates were precleared with mIgG-
25 agarose beads (Sigma) followed by incubation with Protein A-purified polyclonal rabbit antibody specific for soluble attractin or Protein A-purified normal preimmune IgG. Antibody complexes were isolated by incubation with agarose beads conjugated with antibody specific for
30 rabbit IgG (Sigma) followed by boiling in 2x SDS-PAGE loading buffer, after which the procedure was identical with that described for Western transfers above.

For stable transfections, pSecTag2B-soluble attractin-1 was introduced into CHO cells by
35 electroporation (250V, 1600 μ F) using the Cell-Porator

T 0 2 3 3 9 " 2 5 0 4 0 4 5 0

- 40 -

apparatus (Life Technologies) and selection with zeocin (500 µg/ml; Invitrogen). To purify recombinant soluble attractin-1, cells were lysed and loaded onto a Talon Superflow metal affinity resin (Clontech) and eluted with 5 250 mM imidazole. DPPIV activity of the recombinant soluble attractin-1 was determined using gly-pro-pNA as substrate as described previously [Duke-Cohan et al. (1995) *J. Biol. Chem.* 270, 14107-14114].

Binding assays: PBMC were activated for 24 hr 10 with PHA in AIM V medium as described above, washed in AIM V and 10^6 cells/100 µl were incubated for 1 hr at 4°C with doubling dilutions of 125 I-labeled soluble attractin [Duke-Cohan et al. (1996) *J. Immunol.* 156, 1714-1721], starting at 2 µg/ml (10^7 dpm/µg). The cells were washed 15 with cold PBS, and the pellet and first wash supernatant were counted by scintillation. Results were analyzed by Scatchard analysis.

Electron microscopy: Cells were prepared as described previously [Xu et al. (1994) *J. Histochem. Cytochem.* 42, 1365-1376] and were analyzed by 20 transmission electron microscopy (model JEM 100 CX II; JEOL, Peabody, MA).

Example 2. Soluble Attractin-1 Binds Strongly to T Cells and Induces Spreading of Monocytes.

25 Purified serum (soluble) attractin-1 had previously been found to enhance the proliferative responses of PBMC to recall antigens such as tetanus toxoid. In the absence of the antigen, the soluble attractin-1 had no effect. Scatchard analysis showed 30 that about 1,000 molecules of soluble attractin-1 bound to the surface of a resting T cell, and about 2,000 molecules to a PHA-activated T cell, with a K_d for both of between 5 and 50 pM which is indicative of a specific, high affinity interaction. To determine whether the

T02290" 062704

- 41 -

binding of soluble attractin-1 had a functional effect upon cells, a population of PBMC was tested for morphological changes due to the presence or absence of soluble attractin-1. It was found that, within 48 hr of addition to PBMC in serum-free medium, soluble attractin-1 caused spreading of adherent macrophage-like cells, generating long processes to which lymphocytes attached. This process was dose-dependent, with the maximum effect occurring between 5 to 10 μ g/ml soluble attractin-1 (Figs. 1A-1F). Soluble attractin-1 had no effect on E⁻ lymphocytes alone (Fig. 1G) or E⁻ monocytes/B lymphocytes alone (Fig. 1H), but clustering occurred if the E⁺ T and E⁻ populations were combined (Fig. 1I). After non-adherent cells had been washed away, the adherent cells were released by incubation with EDTA in PBS. By using immunofluorescence analysis, it was found that the small adherent cells were exclusively CD3⁺ T cells, while the large adherent cells were predominantly CD14⁺ monocytes/macrophages.

20 Example 3. Cloning of cDNA Encoding Soluble Attractin-1 and Analysis of the Deduced Protein Structure.

Peptide sequences within the soluble attractin-1 polypeptide were identified with a view to cloning cDNA encoding it. Natural soluble attractin-1 was purified to homogeneity and the N-termini of 16 proteolytic peptides (underlined in Fig. 2) were sequenced. One of the sequences (17 amino acids) was 100% identical to part of the derived amino acid sequence of a translated 3' EST sequence (R84298) which codes for 1.9 kb sequence including the 3' end of soluble attractin. By using this sequence as a probe, two mRNA species of 4.4 kb and 8-9 kb were detected, both of which were heavily represented in fetal liver and spleen (Fig. 3A). The larger form was dominant in thymus while the smaller form was dominant in

- 42 -

PBMC. No upregulation of attractin mRNA transcription was observed in activated T cells (Fig. 3B).

Based on mRNA expression, a fetal liver library was screened, and a 982 bp 5' fragment derived from the longest clone (pks43) was used to rescreen the library, leading to identification of a further clone containing additional 5' sequence (pks43-1). Sequencing of both overlapping clones yielded an open reading frame (ORF) of 3.594 kb that encoded all 16 peptides previously identified. PCR amplification of the main body of soluble attractin-1 cDNA from the two activated T cell libraries and from the fetal liver library produced PCR products of the same size, i.e., about 3 kb (Fig. 3C). The nucleotide sequence coding for soluble attractin-1 (SEQ ID NO:2) has been deposited in GenBank and given the accession number AF034957.

The codon encoding the first methionine of soluble attractin-1 is within a consensus Kozak sequence, and the subsequent ORF codes for a 134 kDa protein with 26 potential N-glycosylation sites. Although soluble attractin-1 is heavily glycosylated, no consensus leader sequence/signal peptides could be identified. Several distinct domains and motifs can be identified in the ORF, as depicted in Fig. 4B. These include a serine (Ser-26) within a hybrid of prolyl oligopeptidase and trypsin-like serine protease catalytic motifs (Fig. 4C), an EGF domain (Gly-24 to Gln-54), a CUB domain (His-57 to Phe-173), an EGF domain (Met-175 to Ala-207), the ligand-binding motif of the common γ cytokine chain (Cys-636 to Trp-648), a C-type lectin domain (Ile-713 to Cys-844), and 2 cysteine-rich regions incorporating the C-terminal laminin-like EGF domains (Ala-988 to Lys-1031 and Pro-1034 to Cys 1066). Fragments of soluble attractin-1 containing one or more of these domains are within the invention, as are nucleic acids encoding such fragments.

- 43 -

The only highly significant match at both the nucleotide and amino acid level (31% identity, 45% similarity across the complete 1198 amino acids) is with the nematode F33C8.1 perlecan-like protein, which has an identical organization of the CUB and EGF domains and conserved cysteine positioning suggestive of a similar secondary structure in the two proteins (Fig. 4B). In contrast to soluble attractin-1, F33C8.1 does not express the γ chain binding motif or the C-type lectin domain.

10 Soluble attractin-1 appears to be a new member of the CUB domain family of proteins, initially consisting of the complement proteins C1r/C1s, Uegf and BMP-1 [Bork et al. (1993) *Mol. Biol.* 231, 539-545].

15 Example 4. Subcellular Localization and Synthesis of the Soluble Attractin Molecule.

To understand the secretory route followed by soluble attractin-1 in the absence of a signal peptide, we determined the subcellular localization of soluble attractin-1 in resting and activated E⁺ T lymphocytes. No glycosylated soluble attractin-1 could be detected anywhere in resting T cells (Fig. 5A), whereas in T cells activated for 48 hr with PHA, soluble attractin-1 was clearly localized in large vesicular structures (Fig. 5B) that often contained an electron-dense core (Fig. 5C).

25 Vesicles containing soluble attractin-1 were often clustered close to the plasma membrane where they released soluble attractin-1 into the extracellular space (Fig. 5D). Soluble attractin-1-encoding cDNA was cloned into the expression vector pRc/CMV which was transfected into CHO cells. Glycosylated soluble attractin-1 could not be detected in the transfected CHO cells. The post-translational glycosylation of soluble attractin-1 was "forced" by cloning soluble attractin-1 cDNA into the pSecTag2B expression vector which supplies a N-terminal

- 44 -

leader sequence. In an *in vitro* transcription/translation system, pSecTag2B-soluble attractin-1 produced a protein of 134 kDa and in the presence of glycosyl transferases yielded a product of about 180 kDa (Fig. 6A). The correctly sized product was also detected by Western blotting, with antibody specific for myc, of a whole cell lysate of 293T cells transiently transfected with pSecTag2B-soluble attractin-1 (Fig. 6B). The polyclonal antibody specific for soluble attractin immunoprecipitated recombinant soluble attractin-1 from CHO cells stably transfected with pSecTag2B-attractin, confirming that the overall structure of the recombinant protein was similar to that of the purified natural material (Fig. 6C).

Example 5. Functional Activities of Recombinant Soluble Attractin-1.

Soluble attractin-1 was isolated from lysates of stably transfected CHO cells. Even with a signal peptide, the recombinant attractin localized intracellularly and was not secreted. The DPPIV enzyme activity of the recombinant protein was 0.42 units/mg, in comparison with 0.79 units/mg for T cell-released soluble attractin-1, 1.78 units/mg for serum attractin, and 4.12 units/mg for recombinant CD26.

The PBMC interaction assays depicted in Fig. 1 were repeated using recombinant rather than natural serum-purified soluble attractin-1. This experiment showed that the spreading effect of recombinant soluble attractin-1 on monocytes/macrophages was, as in the experiments with natural serum-purified soluble attractin-1, dose-dependent (Figs. 7A-7D). At a concentration of 5 μ g/ml, the effect was similar to that of 5 μ g/ml natural soluble attractin-1 and thus confirmed the results observed with purified natural soluble

- 45 -

attractin-1. There was a greater tendency for clustering of T cells when the recombinant soluble attractin-1 was used. However, these clusters were not proliferating cells, as indicated by the fact that no increase in [³H]-thymidine incorporation could be detected over background in cultures of PBMC containing recombinant soluble attractin-1.

Example 6. Evidence for Involvement of Attractin in Human Immune Responses.

10 Clinical studies were performed in order to investigate whether attractin plays a role in the human immune system *in vivo*. Common variable immunodeficiency (CVI) is a late-onset primary immunodeficiency affecting either humoral or cellular immunity. The mechanism
15 underlying this disease is still unknown. In a normal immune response, the surface expression of attractin is upregulated during T cell activation. The early signaling events during T cell activation were studied in 11 patients (age range: 7-27 years) affected by CVI to
20 determine if the expression pattern of attractin is different from that of normal individuals. Cell-surface activation markers, including attractin, were evaluated on resting or 24-48 hour CD3-activated T cells by dual color fluorescence. In cells from all patients but one,
25 in contrast to those from normal control subjects, the T cell surface expression of attractin was not upregulated after CD3 crosslinking. The lack of attractin upregulation was selective in that an increase of the other activation markers was observed in the CVI
30 patients.

T04299" 6028260

- 46 -

Example 7. Production of Single Chain Variable Region
Fragment (scFv) Antibodies Specific for Attractin.

Initial efforts to produce murine monoclonal antibodies were unsuccessful, apparently as a direct
5 consequence of the high degree of conservation between
the mouse sequence identified to date and the human
sequence for attractin. To overcome the problem of
antigen conservation across species, techniques have been
developed for cloning antigen-binding regions of V^H and V^L
10 genes from the mRNA of non-immune spleen cells,
connecting the variable regions with a flexible peptide
linker to produce single chain variable region fragments
(scFv). The scFv are then expressed as fusion proteins
with phage coat protein [Sheets et al. (1998) Proc. Natl.
15 Acad. Sci. USA. 95, 6157-6162] in phage particles. Using
this technique, it is possible to generate repertoires of
10⁷ to 10¹⁰ scFv variants. The reagents for generating a
library and subsequent enrichment of murine antigen-
specific scFv are now available in kit form from Amersham
20 Pharmacia Biotech.

Three separate libraries are simultaneously
produced from murine spleen cells. The first library is
produced from mRNA of non-immune spleen cells, the second
from spleen cells of mice which have received prior
25 immunization with glycosylated native attractin, and a
third library from spleen cells of mice which have been
immunized with deglycosylated attractin. Messenger RNA
is extracted from the lysed spleen cell populations by
hybridization to oligo dT cellulose and first strand cDNA
30 is synthesized using M-MuLV reverse transcriptase. Using
primers designed to anneal to the 5' and 3' ends of
murine heavy chain and kappa light chain variable region
sequence, the repertoire of expressed variable regions
sequences is amplified by PCR. Lambda light chain
35 sequences are not amplified as they represent only a

03767097 062701

- 47 -

small fraction of mature antibody-expressed light chain, and the flanking sequences are variable. The PCR products from the heavy chain amplification (≈ 400 bp) and the kappa light chain amplification (≈ 300 bp) are
5 separated by agarose gel electrophoresis, gel plugs cut out, and the DNA extracted by freezing and thawing followed by membrane centrifugation.

A linker that hybridizes to the 3' end of the heavy chain and the 5' end of the light chain and encodes
10 the peptide liner (e.g., Gly₄Ser₃) is then used to amplify out a full length scFv region of 750 bp. Through use of 5' and 3' primers which carry 5' tails encoding restriction endonuclease sites, Sfi I and Not I sites are added by PCR to the 5' and 3' termini, respectively, of
15 the scFv. After digestion with Sfi I followed by Not I, the phenol/chloroform-precipitated fragments are directionally ligated into the pCANTAB 5E phagemid and the construct encoding multiple scFv is used to transform *E. coli* TG1 cells. The transformed cells are then
20 infected with M13K07 helper phage to rescue the phagemid which will result in expression of the recombinant scFv as a fusion protein based on the gene III phage protein, the fusion protein being displayed on the phage particles.

25 At this point, the scFv-expressing phages are panned on attractin-coated plates, unbound phages are washed away, bound phage eluted, the phage DNA isolated, and TG1 cells reinfected and allowed to express by superinfection with helper phage. The panning process is
30 repeated up to 3 times in order to refine the specificity. Once antigen-reactive clones have been identified, they are tested for binding by adding the scFv-expressing phage to attractin-coated wells, washing away unbound phage, adding horseradish peroxidase
35 conjugated antibody specific for M13 phage, washing away

"062701" 062701 062701

- 48 -

unbound conjugate and adding ABTS substrate. After confirmation of specific binding by ELISA, the recombinant phage is used to infect *E. coli* HB2151, which recognizes the amber stop codon encoded by pCANTAB 5E, allowing production of soluble scFv not in the context of the phage gene III protein. The soluble scFv can be isolated from *E. coli* periplasm using standard procedures and the sample can then be applied to an affinity column bearing antibody directed against a peptide region (-Gly-Ala-Pro-Val-Pro-Tyr-Pro-Asp-Pro-Leu-Glu-Pro-Arg-Ala-Ala-COOH (SEQ ID NO:9)) downstream from the kappa light chain and which generates a C-terminal common to all the scFv fragments. Binding occurs at neutral pH and, comparable to a normal antigen-antibody reaction, can be eluted at acidic pH. This peptide can be recognized by its specific antibody under native and denatured conditions. scFv produced in this way will provide a very specific handle for studying T lymphocyte intracellular expression and surface expression of attractin, for determining the kinetics of surface expression during clustering, for blocking functional clustering assays, and for determining both levels of attractin released from T cells during assays in vitro and levels in biological fluids.

In addition to maximizing selection of antibodies with the greatest affinity, panels of scFv directed against specific epitopes of attractin are developed. This is achieved by taking all isolated attractin-specific scFv and repeating the ELISA assays described above using the above-described forms of attractin and attractin deletion mutants as the target antigen. Selective binding of the scFv to one form of attractin but not to another or the deletion mutants will indicate that the scFv is specific for the relevant form of

- 49 -

attractin or the deleted part of the polypeptide, respectively.

Example 8. Identification of the Nucleotide and Amino Acid Sequence of Membrane Attractin-1

5 Initially, a 9 kb murine mRNA was shown to encode a molecule which was similar to human attractin-1. Sequence analysis of murine cDNA revealed that the nucleotide sequence diverged, extended the open reading frame, and coded for a transmembrane domain and a
10 cytoplasmic domain. Given the 93% identity in the amino acid sequences of the regions shared by human soluble attractin-1 and murine attractin, it was hypothesized that an oligonucleotide probe based on the region coding for the murine cytoplasmic tail would also hybridize to a
15 human mRNA that coded for an attractin with a cytoplasmic tail. This was found to be the case. Such a probe hybridized to the 9 kb human attractin mRNA but not to the 4.5 kb form, while a probe based on sequence coding for the common N-terminal sequence hybridized to both
20 forms. This indicated that the 9kb human mRNA coded for a long attractin similar to the murine molecule. Searching of the EST database then revealed a deposited human sequence (KIAA0548; GenBank AB011120) that coded for 451 amino acids at the C-terminal of a human membrane
25 attractin. The identification of this sequence re-enforced the hypothesis that a natural mRNA for membrane attractin existed. Using KIAA0548 as a base, a human genomic clone that contained an apparent attractin exon corresponding to the sequence coding for the amino acid
30 sequence CEVENRYQGNPLRGTCY (SEQ ID NO:20), close to the C-terminal of attractin, was identified. Complete sequencing of this genomic clone proved conclusively that the divergence between soluble attractin-1, and membrane attractin-1, was the result of alternate splicing.

05767097 "062704

- 50 -

The nucleotide sequence of cDNA encoding human membrane attractin-1 (SEQ ID NO:11) is shown in Fig. 10 and the amino acid sequence of the human membrane attractin-1 protein (SEQ ID NO:10) is shown in Fig. 9.

5 Note that the C-terminal five amino acids of soluble attractin-1 (SEQ ID NO:2 in Fig. 2) differs from the equivalent five amino acids in membrane attractin-1 and it lacks an additional 156 C-terminal amino acid residue region containing a transmembrane domain (amino acid

10 residues 1205-1225) domain and a cytoplasmic domain C-terminal to the transmembrane domain.

Example 9. Deduction of the Attractin-2 cDNA and Protein Sequences.

Multiple attractin cDNA species covering the 5' region have been identified which include or do not

15 include a 222-bp insertion encoding a 74 amino acid region that defines attractin-2 proteins. Since there is no reason to suppose that this region can in any way influence 3' splicing events, it is likely that both

20 membrane and soluble attractin mRNA species containing the 222-bp insertion are generated. Further evidence that such transcripts exist comes from experiments with mouse mRNA from which it is clear that there are multiple mouse attractin mRNA species of which the membrane form,

25 at least, contains a 72 amino acid (216 bp) insertion corresponding to the 74 amino acid insertion of human attractin-2. Thus, the invention includes both soluble attractin-2 (SEQ ID NO:18) (Fig. 11), membrane attractin-2 (SEQ ID NO:12) (Fig. 13) and the cDNA sequences

30 encoding soluble attractin-2 (SEQ ID NO:19) (Fig. 12) and membrane attractin-2 (SEQ ID NO:13) (Fig. 14). The transmembrane domain of membrane attractin-2 is predicted to include residues 1279-1301 of SEQ ID NO:12 and its

05707097.062704

- 51 -

cytoplasmic domain is predicted to include all residues C-terminal of the transmembrane domain.

Example 10. Recombinant Soluble Attractin-1 Increases Expression of MHC Class II and B7.1 Molecules on

5 Monocytes.

Peripheral blood leukocytes were isolated (from blood donated by normal donors in the Blood Bank of the Dana Farber Cancer Institute) by centrifugation over Ficoll-Hypaque. The isolated cells were suspended in
10 defined lymphocyte culture medium (AIM V; GIBCO-BRL) at a concentration of 2×10^6 per ml and incubated at 37°C in an atmosphere of 7.5% CO_2 for 72 hr with soluble recombinant attractin-1 (at 5 ug/ml) or with GM-CSF (10 U/ml). Non-adherent cells were then discarded and adherent cells
15 were recovered using PBS containing 5 mM EDTA and washed in AIM V medium. All cells were suspended at a concentration of 10^7 per ml. 100 ul aliquots (corresponding to 10^6 cells) were incubated with 0.25 to 1 ug of a panel of FITC- or phycoerythrin-labelled
20 antibodies directed against a selection of known pan-leukocyte, T cell-specific, B cell-specific, monocyte-specific and NK-specific markers. Using the Coulter XL fluorescence analysis machine, fluorescence windows were gated on the total cell population, the lymphocyte
25 population, and the monocyte population that is distinguished by size and light "scatter" (Fig. 15). The profiles with dark fill were obtained with control cells incubated with antibodies of irrelevant specificity conjugated with appropriate fluorophores, and those with
30 light fill were obtained with cells incubated with antibodies with the indicated specificity. Also shown in Fig. 15 are similar data obtained with peripheral blood leukocytes prior to culture (D0).

- 52 -

Monocytes incubated with GM-CSF differentiated by losing CD14 (Fig. 15) and expressing CD1a (not shown), indicating a differentiation towards early dendritic cells. For monocytes incubated with soluble attractin-1 in the presence of CD3+ T lymphocytes, CD14 remains high, there is no induction of CD1a (not shown), B7-1 expression begins to increase, and MHC Class II expression increases off scale (Fig. 15). These findings indicate that attractin serves to enhance the antigen presenting function of monocytes.

While the present invention has been described in conjunction with a preferred embodiment, one of ordinary skill, after reading the foregoing specification, will be able to effect various changes, substitutions of equivalents, and other alterations to the compositions and methods set forth herein. It is therefore intended that the protection granted by Letters Patent hereon be limited only by the definitions contained in the appended claims and equivalents thereof.

T 0 3 3 0 3 6 0